ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 10, Issue 8, 2017

Online - 2455-3891
Print - 0974-2441

Research Article

IN VITRO ANTIMYCOTIC EVALUATION AND PRELIMINARY COMBINATORIAL STUDIES OF IBUPROFEN WITH STANDARD ANTIFUNGAL DRUGS AGAINST *ASPERGILLUS* SPP.

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Received: 31 March 2017, Revised and Accepted: 21 April 2017

ABSTRACT

Objective: The prevalence of invasive mycoses is increased in the immunocompromised patients with an increase in resistance developed against current antifungal drugs. This has led to the need for discovering novel combinations of the antifungal drugs to combat against resistant pathogenic spp. This study mainly targets to evaluate the antifungal activity of ibuprofen (IBU) alone and in combination with the standard antifungal drugs (polyenes and azoles) against eight isolates of *Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus niger*.

Methods: The study was performed using the disc diffusion assay (DDA), microbroth dilution assay and spore germination inhibition assay. Moreover, cytotoxicity was checked by heamolytic assay.

Results: Minimum inhibitory concentration (MIC) of IBU against *A. fumigatus* and *A. flavus* using DDA is found to be in the range of 250-275 µg/disc while for *A. niger* isolates, the range was 500-575 µg/disc. Likewise, by broth microdilution assay and spore germination inhibitory assay, MIC determined, were in the range of 500-750 µg/ml against *A. fumigatus* and *A. flavus* while for *A. niger,* it was 1000-1500 µg/ml.

Conclusion: IBU demonstrated its antimycotic potential against all the eight isolates of *Aspergillus* spp. Moreover, preliminary combinatorial evaluation of IBU with the standard antifungal drugs reported by DDA revealed an increase in zone of inhibition as compared to the drugs alone. Further research regarding the confirmation of synergistic interaction between the selected drugs is in progress.

Keywords: Aspergillosis, Antifungal, Combination, Ibuprofen.

 DOI: http://dx.doi.org/10.22159/ajpcr.2017.v10i8.18188

INTRODUCTION

A prevalence of the opportunistic mycotic infections has varied for the past few decades. Invasive aspergillosis (IA), predominantly caused by *Aspergillus fumigatus*, followed by *Aspergillus flavus* and *Aspergillus niger*, have increased the risk of morbidity and mortality among immunosuppressed cases [1,2]. Although, for the past few decades, antimycotic agents such as polyenes, azoles, and echinocandins have proved to act significantly against IA [3,4]. However, the resistances against these standard antimycotic agents have developed the urge to search for the new drugs that can help eradicate severe opportunistic mycotic infections like IA. Moreover, lack of the effective monotherapy and the combination therapy of the standard drugs against IA has further allowed the researchers to develop new combinations that can lead to better therapeutic treatment for IA.

Ibuprofen (IBU) is a derivative of the propionic acid and relates to the class of non-steroidal anti-inflammatory drugs (NSAID), known for its anti-inflammatory, analgesic and anti-pyretic properties [5]. IBU inhibits the synthesis of prostaglandins from the arachidonic acid as it has the capability to obstruct the cyclo-oxygenase enzyme [5,6]. Earlier studies have demonstrated the antifungal activity of IBU and its synergy with the azoles against *Candida* spp. [7,8]. In addition, the IBU has also been reported to induce blocking of the efflux pumps such that reverting resistant *Candida* spp. into susceptible ones, against azoles [9,10].

Thus, in this study, antifungal activity of IBU has been evaluated alone and in combination with standard antifungal drugs, the polyenes: Amphotericin B (AmpB), nystatin (NYST) and the azoles: Ketoconazole (KETZ), flucanozole (FLUZ), via., various techniques against three clinical isolates of *A. fumigatus,* three of *A. flavus* and two clinical strains of *A. niger*.

METHODS

The antifungal drugs, AmpB, NYST, KETZ, and FLUZ obtained from Himedia Chemicals and IBU from the Fluka Analytical Chemicals. 4 mg/ml AmpB and 10 mg/ml of IBU were taken as the stock solution and diluted with the distilled water to get the required concentration depending on the experiment done.

Pathogens

Pathogenic strains of *A. fumigatus* (clinical isolate 190/96 [Vallabhbhai Patel Chest Institute, Delhi (VPCI) and ITCC1634 [Indian Agricultural Research Institute Delhi (IARI)] and ITCC 4517 [IARI, Delhi]), *A. flavus* (clinical isolate 223/96 [VPCI, Delhi], ITCC 5192 [IARI, Delhi], and ITCC 5076 [IARI, Delhi]), and *A. niger* (clinical isolate 56/96 [VPCI, Delhi] and ITCC 5405 [IARI, Delhi]) were used in the current research. All the isolates were grown on sabouraud dextrose (SD) agar culture Petri plates of 10.0 cm diameter (tarsons) by inoculating with stock of *A. fumigatus*, *A. flavus,* and *A. niger* and incubating at 37°C in a biochemical-oxygen demand incubator. After 96 hrs incubation, spores were collected from culture plates and distributed evenly in phosphate buffer saline (PBS). The spores were collected and a concentration of 10^8 spores/mL was used to perform the experiments.

Assay

The antimycotic activity of IBU was analyzed by the disc diffusion assay (DDA), microbroth dilution assay (MDA), and spore germination inhibition assays (SGIA) as illustrated previously [4,11].

DDA

DDA was performed using the radiation sterilized Petri plates cultured with the SD agar and inoculated with 100 µl of homogenized suspension of 1×10^8 spores/ml of *Aspergillus* spp. Discs of 5.0 mm in diameter (Whatman filter paper No. 1) were placed on dried plates. Furthermore, discs were impregnated with the different concentrations of IBU in the range of 125-1000 µg/disc and AmpB within range of 5-0.35 µg/disc. After incubation at 37°C, Petri plates were observed at 24 and 48 hrs to check if there was any zone of inhibition, around the discs to know the antifungal effect.

MDA

The assay was executed in 96-well microtiter flat bottom plates (Tarson). Different dilutions of IBU starting with 1000 µg/ml were prepared in 100 µl of culture medium. Moreover, wells were administered with 10 µl of spores (1×10^8 spores/ml). 96-well plates were then incubated at 37°C and observed visually after 48 hrs. The well with no growth, i.e., complete inhibition of growth, was taken as end point and considered minimum inhibitory concentration (MIC).

SGIA

Different concentrations of IBU in 100 µl of culture medium were prepared in 96-well plates by double dilution method. Inoculation of each well was done with 10.0 µl of spore suspension containing 100 ± 5 spores. After 16 hrs of incubation at 37°C, microtitre plates were assessed for germination of spores with an inverted microscope. The number of germinated and non-germinated spores will be counted. The percentage of inhibition of spore germination was calculated by the formula described by Ruhil *et. al.* [4]. The least concentration of the IBU, which was responsible for more than 90% inhibition of spore germination was taken as MIC_{90}.

Toxicity studies

Haemolytic assay

This method [4,11] was performed using the erythrocytes from the healthy humans was taken and rinsed them with autoclaved PBS. Erythrocyte suspension (2.0% v/v) was prepared and exposed with IBU and AmpB starting with concentration of 1000 µg/ml at 37°C for 1 hr. The eppendorph tubes were allowed to centrifuge at 5000 rpm (rotation per minute) for 10 minutes. Optical density of the supernatant was calculated at 415 nm by spectrophotometer (Ultraviolet–visible Spect Lambda Bio 20 Perkin-Elmer). Hemolysis done by the test compounds were measured in percentage and the concentration responsible for <10% hemolysis was considered non-toxic. PBS was used as the negative controls while triton X as positive controls along with the erythrocytes.

***In vitro* combinatorial evaluation**

In vitro combinatorial evaluation (preliminary) among polyenes and azoles with NSAID IBU was performed using DDA. The 6 mm discs of Whatmann filter paper one were inoculated with 1.1 µg/disc of AmpB, NYST, KETZ, FLUZ, and MIC of KETZ and FLUZ, as calculated by DDA) alone and with 129 µg of IBU. Incubating the plates at 37°C for 48 hrs allowed the zone of inhibition to be visibly clear such that radius was measured after an interval of 24 and 48 hrs. No antifungal potential was declared where zone of inhibition was below 6 mm.

RESULTS AND DISCUSSION

***In vitro* antimycotic potential**

The MIC of AmpB, KETZ, FLUZ, NYST by using DDA was 0.76, 6.24, 31.26, and 1.1 µg/disc, respectively, against all the eight pathogenic stains of *Aspergillus* genera. The mean of diamtere of zone of inhibition along with standard deviation was also measured for IBU as shown in Table 1.

Remarkably, IBU created an optically clear zone of inhibition of mycotic growth against all eight *Aspergillus* isolates. The geometric mean (GM) MIC of AmpB (standard antifungal), against three isolates of *A. fumigatus, A. flavus* and two of *A. niger* was 1.6 µg/ml, by MDA and SGIA. The GM of MIC of IBU has been summarized in Table 2.

The antifungal activity of IBU against the most pathogenic strain, i.e., *A. fumigatus* (VPCI190/96) was clearly noticeable with DDA, MDA, SGIA shown in Figs. 1-3. These outcomes established that IBU have natural activity against the *Aspergillus* spp.

The fungal isolates were observed to show disparity in their susceptibility when they were exposed with the IBU in different culture media. The culture media can influence the intensity of antimycotic activity [12]. Consequently, *in vitro* activity of the IBU against all the eight isolates of *Apergillus* spp were examined in three test media, i.e., RPMI 1640, potato dextrose broth (PD broth) and SD broth, concluded in Table 3.

Toxicity test

Complete lysis of erythrocytes is done by AmpB, NYST, KETZ at a concentration of 37.30, 67.2, and 124 µg/ml, respectively. However, IBU was non toxic >1 mg/ml (Fig. 4).

Combination study

The polyenes (AmpB and NYST), azoles (KETZ and FLUZ) alone and with IBU was infused on the discs; data have been summarized in Table 4 after calculating zone of inhibition.

The combination of IBU with FLUZ displayed no activity almost or very less potential while increased zone of inhibition reported maximum in a combination of IBU with polyenes and also has synergy with KETZ.

Table 1: MIC determination of IBU by DDA

Drugs	Mean of diameter of zone of inhibition		
	Pathogens Mean±SD (mm)		
NSAID	*A.fumigatus* (3)#	*A. niger* (2)#	*A. flavus* (3)#
IBU (µg/disc)			
1000	11.5±0.1	10.4±0.2	11.3±0.2
500	8.9±0.2	7.3±0.3	8.8±0.1
250	6.8±0.1	NAP	6.7±0.2
125	NAP	NAP	NAP
AmpB			
0.76	6.4±0.2	6.5±0.2	6.4±0.1

SD: Standard deivaiton, NAP: No antifungal potential, #Number of strains, NSAID: Non-steroidal anti-inflammatory drugs, IBU: Ibuprofen, MIC:, DDA: Disc diffusion assay, *A. fumigatus: Aspergillus fumigatus, A. flavus: Aspergillus flavus, A. niger: Aspergillus niger,* AmpB: Amphotericin B

Table 2: Summary of GM of MIC of IBU by different assays

Pathogens	GM*MIC of IBU		
	MDA (µg/ml)	DDA (µg/ml)	SGIA (µg/ml)
A. fumigatus (3)	572.35	258.07	572.35
A. niger (2)	1224.74	536.19	1224.74
A. flavus (3)	572.35	258.07	572.35

*GM: Geomatric mean, MIC: Minimum inhibitory concentration, IBU: Ibuprofen, MDA: Microbroth dilution assay, DDA: Disc diffusion assay, SGIA: Spore germination inhibition assays, *A. fumigatus: Aspergillus fumigatus, A. flavus: Aspergillus flavus, A. niger: Aspergillus niger*

Table 3: *In vitro* activity of IBU in different test media

Pathogens	GM MIC of IBU		
	SD broth	PD broth	RPMI 1640
A. fumigatus (3)	572.35	314.980	721.12
A. niger (2)	1224.74	1000	1732.05
A. flavus (3)	572.35	314.980	721.12

IBU: Ibuprofen, GM: Geometric mean, MIC: Minimum inhibitory concentration, *A. fumigatus: Aspergillus fumigatus, A. flavus: Aspergillus flavus, A. niger: Aspergillus niger,* PD broth: Potato dextrose broth

Table 4: *In vitro* combination of the standard drugs with IBU against the *Aspergillus* spp. by DDA

Drugs (µg/disc)	Mean diameter zone of inhibition (mm)					
	Pathogens					
	A. fumigatus (3)		*A. niger* (2)		*A. flavus* (3)	
	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs
AmpB (1.1)	7.3±0.1	7.2±0.2	6.8±0.2	6.5±0.1	7.3±0.1	7.2±0.2
NYST (1.1)	6.9±0.1	6.4±0.2	6.5±0.2	6.1±0.1	6.9±0.1	6.4±0.2
KETZ (6.24)	6.4±0.2	6.2±0.1	6.3±0.1	6.0±0.1	6.4±0.2	6.2±0.1
FLUZ (31.26)	6.2±0.2	6.0±0.2	6.1±0.1	NAP	6.2±0.2	6.0±0.2
IBU (129)	NAP	NAP	NAP	NAP	NAP	NAP
AmpB:IBU (1.1:129)	12.8±0.1	12.6±0.2	12.0±0.1	11.8±0.2	12.8±0.1	12.6±0.2
NYST:IBU (1.1:129)	7.8±0.1	7.6±0.2	7.3±0.1 7	2±0.1 7	8±0.1	7.6±0.2
KETZ:IBU (6.24:129)	7.0±0.2	6.8±0.3	6.9±0.1	7.1±0.1	7.0±0.2	6.8±0.3
FLUZ:IBU (31.26:129)	6.5±0.2	6.4±0.3	6.3±0.2	6.2±0.2	6.5±0.2	6.4±0.3

NAP: No antifungal potential, *A. fumigatus: Aspergillus fumigatus, A. flavus: Aspergillus flavus, A. niger: Aspergillus niger,* IBU: Ibuprofen, AmpB: Amphotericin B, NYST: Nystatin, KETZ: Ketoconazole, FLUZ: Flucanozole, DDA: Disc diffusion assay

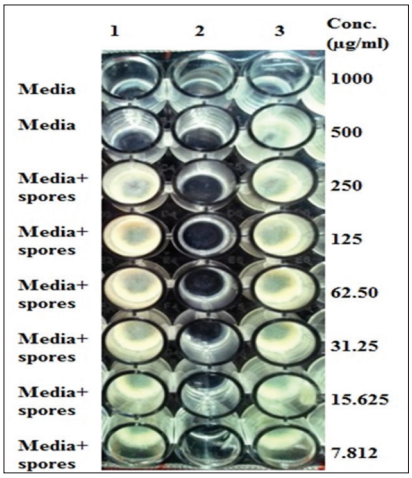

Fig. 1: Microbroth dilution assay of ibuprofen against *Aspergillus fumigatus* (Vallabhbhai Patel Chest Institute, 190/96)

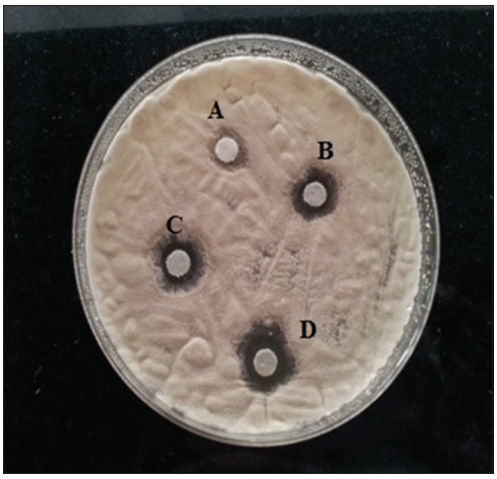

Fig. 2: Disc diffusion assay of non-steroidal anti-inflammatory drugs (ibuprofen) against *Aspergillus fumigatus* (Vallabhbhai Patel Chest Institute, 190/96). A - 125 µg/disc, B – 250 µg/disc, C – 500 µg/disc, D - 1000 µg/disc

These data suggest that IBU have positive interactions with polyenes and KETZ.

Our results clearly portrayed that NSAID can indisputably be administered as antimycotic agent to boost efficiency of current

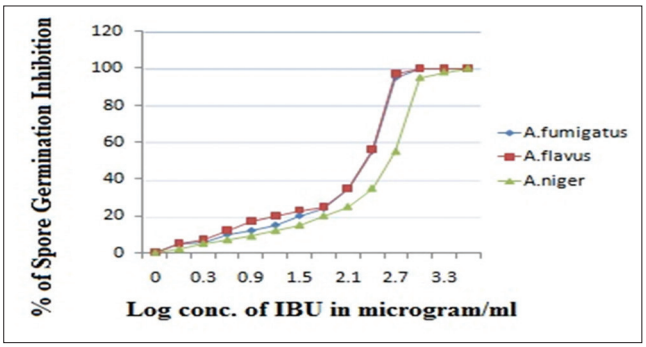

Fig. 3: Spore germination inhibition assays of ibuprofen against *Aspergillus* spp.

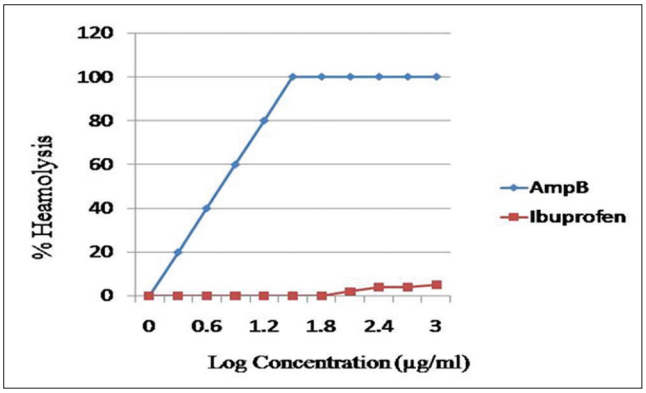

Fig. 4: Toxicity test of ibuprofen by heamolytic assay

antimycotic drugs as IBU is non-toxic even at higher concentrations than standard antifungals so there cytotoxicity might get reduced while administered in combination.

CONCLUSION

Constant investigations for proficient prevention of IA have been in progress for the past few decades. As a result, alternative drugs whose antifungal properties are still unknown can be tested against pathogenic opportunistic mycotic agents such as IBU. Their interaction with the standard antifungal agents (polyenes and azoles) reduced the toxic effect associated with the current drug monotherapies while increasing their antimycotic activity. Our study displayed synergistic and effective interaction between NSAID IBU and polyenes (maximum). Further research regarding confirmation of synergistic interactions between the selected drugs is in progress.

ACKNOWLEDGMENTS

Mrridula *et al*. would like to thank Centre for Biotechnology, Maharshi Dayanand University, Rohtak, India, Rohtak for the necessary financial support.

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